Affidavit of Accuracy

I, Steven Hildebrand, hereby declare that I am fully conversant with the German and English languages and that, to the best of my knowledge and belief, the attached document is a true and accurate translation from German into English of the European Priority Patent Application EP 02005186.8 with the title: "Proteolysis Resistant Active VEGF", on which US Patent Application No.10/506893 is based.

Signed, this 17.day of October, 2007,

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PROTEOLYSIS RESISTANT ACTIVE VEGF

The invention relates to an endothelial growth factor (VEGF) in which the alanine at amino acid (AA) position 111 is replaced by proline and the arginine at AA position 110 can be substituted by another amino acid. The invention also relates to derivatives of the inventive VEGF, nucleic acids, expression systems, medicaments, and the use of the VEGF mutants of the invention for treating chronic wounds.

A large number of mediators which stimulate angiogenesis during wound healing are known. They include firstly the factors which, besides stimulating endothelial cells, also activate mesenchymal and/or epidermal cells (bFGF, aFGF, TGF-*a*, PDGF), and secondly so-called endothelial cell-specific factors whose receptors are substantially confined to endothelial cells (VEGF, angiopoietin). A large number of physiological and pathological reactions involving the blood vessels correlates with an increased expression of VEGF and its receptors, so that VEGF assumes a central role in angiogenesis of the skin. The first indications of the possible importance of VEGF in wound healing impairments were provided on the basis of experiments on VEGF expression in diabetic mice (db/db mice) (Frank *et al.* 1995). It was possible to show in this model that the wound healing impairment correlates with a diminished VEGF expression. It has recently been possible to provide support for the role of VEGF in wound healing by a further transgenic animal model (Fukumura et al., 1998) and detection of VEGF in the wound discharge from acute human wounds (Nissen et al., 1998).

It has further been shown that there is increased expression of the mRNA of VEGF and its receptors in the tissue of chronic wounds (Lauer *et al.*, 2000). Investigations by SDS-PAGE show, however, breakdown of the VEGF protein in the chronic wound environment, in contrast to the acute wound. This breakdown leads to a significant loss of the biological activity and may thus, despite the increased expression of the VEGF receptors, underlie a deficient stimulation of neoangiogenesis in the chronic wound environment. As explained above, it was possible to show that plasmin is involved in the cleavage of VEGF in the chronic wound environment (Lauer *et al.*, 2000).

Cleavage of $VEGF_{165}$ via plasmin leads to detachment of the carboxyl-terminal domain which is encoded by Exon 7. Whereas Exons 3 and 4 determine the binding properties of VEGF to the VEGF receptors Flt-l and Flk-l/KDR, Exon 7 has a critical importance in the interaction of VEGF with neuropilin-l (Keyt *et al.* 1996). Neuropilin-l is a 130 kDa cell surface glycoprotein. Its role in the potentiation of the mitogenic effect of VEGF on endothelial cells was described only recently (Soker *et al.* 1998). In this connection, the interaction of neuropilin-l with Flk-l/KDR appears to be important because binding solely of VEGF to neuropilin-l has no signal effect.

Plasmin belongs to the class of serine proteases. These enzymes are able to cleave peptide linkages. The cleavage takes place by a so-called catalytic triad. In the catalytic centre thereof an essential part is played in particular by the eponymous serine, but also by the amino acids histidine and aspartate, because the process of peptide cleavage takes place by means of them (Stryer 1987, pp. 231 et seq.). Although the mechanism of the linkage cleavage is identical in all serine proteases, they differ markedly in their substrate specificity. Thus, plasmin, just like trypsin, cleaves peptide linkages after the basic amino acids lysine and arginine. However, the substrate specificity of plasmin, which is determined by the structure of the catalytic centre, leads to plasmin being unable to cleave all these linkages. Catalysis of peptide-linkage cleavage is possible only if the corresponding protein segments are able to interact with the catalytic centre of the enzyme (Powers *et al.* 1993; Stryer 1987). To date, no unambiguous consensus sequence of a plasmin cleavage site is known.

The present invention is based on the object of providing improved means for healing chronic wounds.

Surprisingly, this object is achieved by the provision according to the invention of a vascular endothelial growth factor (VEGF) variant which is characterized in that alanine at the amino acid position 111 of the VEGF wild-type is replaced by proline. Another embodiment of the invention are also mutants (variants) of VEGF in which arginine at amino acid position 110 is in addition replaced by another amino acid.

The VEGF mutants according to the invention are preferably in the form of one of the splice variants $VEGF_{121}$, $VEGF_{145}$,$VEGF_{165}$,$VEGF_{183}$,$VEGF_{189}$ or $VEGF_{206}$.

The VEGF mutants according to the invention display not only markedly increased stability towards plasmin, but also an activity comparable to that of wild-type VEGF. Surprisingly, the VEGF variants according to the invention additionally display distinctly increased stability in chronic wound fluids.

The mutations have been carried out at a site which is critical for the biological activity of the VEGF molecule. There was thus a fear that a change in the protein structure in this region has a negative effect on the activity of $VEGF_{165}$. The amino acid proline, which is introduced according to the invention at position 111, is a cyclic α-imino acid. Owing to the cyclic form of the pyrrolidine residue, it has a rigid conformation which also has an effect on the structure of the respective proteins. Thus, proline acts for example as a strong α-helix breaker. It is therefore particularly surprising that replacement precisely of alanine at position 111 by proline generates a VEGF mutant which is stable towards the protease plasmin, is stable in chronic would fluids and, at the same time, still has an activity corresponding to that of the wild-type protein.

The invention relates in particular to VEGF variants of the two sequences Seq. No.1 or Seq. No.2.

The invention also relates to variants of the VEGF mutants mentioned above, in which the amino acid sequences are modified or derivatized, or comprise mutations, insertions or deletions. This relates in particular to VEGF variants in which further single amino acids are replaced, and those which are glycosylated, amidated, acetylated, sulphated or phosphorylated. Such VEGF variants preferably have an activity comparable to or higher than the wild-type VEGF.

The VEGF variants according to the invention may also have a signal sequence. The signal sequence may be connected N-terminally to the amino acid chain of the VEGF variant and have the sequence

Met Asn Phe Leu Ser Trp Ser Val His Trp Ser Leu Ala Leu Leu Leu Tyr Leu His His Ala Lys Trp Ser Gln Ala.

The invention also relates to nucleic acids which code for the abovementioned VEGF mutants, and vectors for VEGF expression which comprise such nucleic acids.

The invention relates to a medicament which comprises the abovementioned mutants of VEGF, and to the use of the VEGF mutants for producing a medicament for the treatment of chronic wounds, caused by vascular lesions such as chronic venous insufficiency (CVI), primary/secondary lymphoedema, arterial occlusive disease, metabolic disorders such as diabetes mellitus, gout or decubitus ulcer, chronic inflammatory disorders such as pyoderma gangrenosum, vasculitis, perforating dermatoses such as diabetic necrobiosis lipoidica and granuloma annulare, haematological primary disorders such as coagulation defects, sickle cell anaemia and polycythemia vera, tumours, such as primary cutaneous tumours and ulcerative metastases, and for plasmin inhibition, for inducing neoangiogenesis and/or for inhibiting matrix degradation.

Topical use of growth factors represents a novel therapeutic concept in wound healing. It has been possible to observe an improvement in the healing of chronic wounds in a large number of clinical studies with the use of EGF, bFGF, PDWHF and PDGF (Scharffetter-Kochanek *et al.* 2000). However, a criticism which should be noted is that the results of these studies did not come up to the expectations which existed in view of the good activity of these mediators in animal models (Lawrence *et al.* 1994). This restricted activity of the growth factors is certainly substantially explained by the increased proteolytic activity in the chronic wound environment, which leads to degradation of the topically applied factors. It is thus clear that local wound management by administration of growth factors represents a promising novel therapeutic strategy. However, it is necessary to develop strategies which control the proteolytic activity in the chronic wound environment. The production of master cytokines with increased stability in the chronic wound environment certainly represents a novel therapeutic approach in this connection. The VEGF mutants according to the invention are particularly suitable, because of their high stability in the wound fluid, for the topical treatment of chronic wounds.

EXEMPLARY EMBODIMENT

Mutagenesis:

Four mutants were produced by site-directed mutagenesis by carrying out targeted amino acid replacements at Arg_{110} and Ala_{111}. The cDNA which codes for human $VEGF_{165}$ was

cloned into the SV40 replication expression vector pcDNA 3.1 (from Invitrogen, De Schelp, NL) using the BamHI and EcoRI cleavage sites in the cloning site. The Gene Editor™ system from Promega (Mannheim) was used for the site directed in vitro mutagenesis. This system is based on annealing of oligonucleotides which harbour the appropriate mutation onto the initial sequence. The initial sequence of $VEGF_{165}$ in the region of the mutations is:

106 107 108 109 110 111 112 113
GA CCA AAG AAA GAT AGA GCA AGA CAA G
Pro Lys Lys Asp Arg Ala Arg Gln

To introduce the mutations, the following mismatch oligonucleotides were used as primers:

Mutation 1: Mut_{Ala}:
GA CCA AAG AAA GAT *GCC* GCA AGA CAA G
Pro Lys Lys Asp *Ala* Ala Arg GIn
Mutation 2: Mut_{Gln}:
GA CCA AAG AAA GAT *CAG* GCA AGA CAA G
Pro Lys Lys Asp *Gln* Ala Arg Gln
Mutation 3: Mut_{Pro}:
GA CCA AAG AAA GAT AG*G C*CA AGA CAA G
Pro Lys Lys Asp Arg *Pro* Arg Gln
Mutation 4: $Mut_{Lys\text{-}Pro}$:
GA CCA AAG AAA GAT A*AG C*CA AGA CAA G
Pro Lys Lys Asp *Lys Pro* Arg Gln

The mutagenesis primers used are each detailed with the modified ammo acid sequences obtained therewith. The regions with the bases or amino acids which are changed from the wild-type sequence are in italics.

In mutation 1, $arginine_{110}$ was replaced by a nonpolar alanine. In mutation 2, a polar, uncharged glutamine was introduced at the same position. In mutant 3, the alanine at position 111, not the basic $arginine_{110}$, was replaced by a proline. In mutant 4, two amino

acids were replaced. In this case, lysine and proline were introduced in place of arginine$_{110}$, and alanine$_{111}$. After the mutagenesis had been carried out, the mutations were verified by sequence analysis. The resulting VEGF mutants had the following sequences for amino acids 109-112:

$VEGF_{165}$-wild-type:	-Asp_{109} Arg_{110} Ala_{111} Arg_{112}-
Mut_{Gln}:	-Asp_{109} Gln_{110} Ala_{111} Arg_{112}-
Mut_{Ala}:	-Asp_{109} Ala_{110} Ala_{111} Arg_{112}-
Mut_{Pro}:	-Asp_{109} Arg_{110} Pro_{111} Arg_{112}-
$Mut_{Lys\text{-}Pro}$:	-Asp_{109} Lys_{110} Pro_{111} Arg_{112}-

The mutants Mut_{Pro} and $Mut_{Lys\text{-}Pro}$ are mutants according to the invention, whereas Mut_{Gln} and Mut_{Ala}, are produced and investigated for the purposes of comparison. The resulting $VEGF_{165}$ expression vectors were used in the further investigations.

Production of recombinant $VEGF_{165}$ protein

$VEGF_{165}$ protein was expressed in eukaryotic COS-l cells. The pcDNA 3.1 expression vector used comprises an SV-40 origin of replication. This serves to amplify the vector in cells which express a large T antigen of the SV-40 virus. The COS-l cells used possess a corresponding element integrated into the genome, so that episomal replication of the vector results. Expression of the target protein VEGF for several days is achieved thereby without stable integration (transformation) of the vector into the cell genome. The COS-1 cells were transfected with the expression plasmids obtained in the mutagenesis. For this purpose, the Superfect transfection reagent (QIAGEN, Hilden) was used according to the manufacturer's protocols.

Like a large number of growth factors, $VEGF_{165}$ also has a heparin-binding site which is located at the basic C terminus. The binding to heparin was exploited for purification of the protein by affinity chromatography (Mohanraj *et al.* 1995). The VEGF and VEGF variants were isolated by the following steps:

The COS-1 cells transformed with the expression plasmids were cultivated in serum free DMEM (Dulbecco's modified Eagle's medium) comprising 10% fetal calf serum (FSC), 2 mM L-glutamine, penicillin (10 U/ml) and streptomycin (10 µg/ml) and ITS supplement

(Sigma, Deisenhofen). Conditioned medium (200 ml) was collected after 48 h and incubated with 5 ml of heparin-Sepharose (Pharmacia, Freiburg) at 4°C for 4 hours. The heparin-Sepharose was packed into a column. The later was loaded with culture medium at a flow rate of 25 ml/h. The following steps were carried out:

A: Affinity chromatography with heparin-Sepharose

1. Washing: 0.1 M NaCl; 20 mM Tris/pH 7.2

2. Washing: 0.3 M NaCI; 20 mM Tris/pH 7.2

3. Elution: 0.9 M NaCl; 20 mM Tris/pH 7.2

B: Analysis of the resulting fractions by Western blot analysis

C: Desalting of the VEGF-containing fractions by gel filtration running buffer: 10 mM Tris/pH 7.2

D: Lyophilization of the solution and determination of the concentration by ELISA

The resulting VEGF was investigated by SDS-PAGE. The VEGF protein obtained from COS-l cells differs in its migration behaviour in SDS-PAGE from the commercially available $VEGF_{165}$ protein used (from R&D Systems). In addition to the signal to be detected at 42 kDa (Figure 1, lane 6), a further band with a molecular weight which is a few kDa higher is also evident. The reason for this double band of the VGEF protein expressed in COS-1 cells is an altered glycosylation of the growth factor. On expression of VEGF in COS-1 cells there is formation of two differently glycosylated proteins. One form (42 kDa) is identical in its glycosylation to the recombinant $VEGF_{165}$ which has been used to date and which was produced in insect cells using a baculovirus expression system (R&D Systems, Figure 1, lane 1). It has an N-glycosylation on the amino acid asparagine at position 74 (Gospodarowicz *et al.* 1989; Keck *et al.* 1989). The second band at a higher molecular weight (45 kDa) results from further glycosylation of the protein. The difference in the glycosylation is known for expression in COS cells and has no effect on the biological activity of the growth factor (R&D Systems).

Characterization of the biochemical and biological properties of the purified $VEGF_{165}$ proteins

I. Analysis of the stability o the $VEGF_{165}$ proteins and its mutations:

a) Incubation in plasmin

The four purified mutated VEGF proteins were initially investigated for their stability towards the protease plasmin. It was investigated whether the mutations carried out lead to an altered degradation behaviour compared with wild-type VEGF.

Figure 1 shows the results of incubation of the VEGF wild-type and the VEGF mutants with plasmin. Incubation of the VEGF wild type synthesized in COS-1 cells (A, lane 6-10) shows degradation of the growth factor after only 15 minutes. In this case, accurate determination of the size of the resulting fragments by SDS-PAGE is difficult because the signals overlap with the two bands of the differently glycosylated protein. However, the degradation pattern is similar to that of the commercially obtainable $VEGF_{165}$ (Figure lA, lane 1-5). Thus, a fragment with a molecular weight of 38 kDa can be detected after 45 minutes. This corresponds to the 110 dimer fragment of the less glycosylated VEGF variant. These results clearly show that the VEGF protein expressed in the COS-1 cells is also cleaved by plasmin under the chosen conditions.

Figure 1B (lane 1-17) shows the results of incubation of mutated proteins. Incubation of the arginine to alanine mutation is shown first (lane 1-5). At zero incubation time, two bands are detectable for the differently glycosylated variants of the VEGF protein, as with the wild type. However, in this case, because of the higher signal intensity, they cannot be differentiated from one another so clearly as with the $VEGF_{165}$ wild-type. In contrast to the VEGF wild-type, the mutated protein shows no change in the migration behaviour up to 240 minutes after incubation.

This observation suggests that the $argimne_{110}$, to $alanine_{110}$, mutation has led to inactivation of the plasmin cleavage site. As shown further in Figure 1B, the three other mutants Mut_{Pro}, Mut_{Gln} and $Mut_{Lys\text{-}Pro}$ also show a comparable stability of the signal bands at 45 and 42 kDa after incubation with plasmid for 240 minutes. A control in which the $VEGF_{165}$ wild-type was incubated with plasmin buffer at 37°C for 4 hours is not degraded (lanes 18 and 19). Overall, these experiments indicate that the produced and purified VEGF mutants are stable towards the protease plasmin.

b) Incubation in acute and chronic wound fluid

In the next step, the degradation of the VEGF mutants in wound fluid from patients with acute and chronic wounds was analysed. On incubation of the $VEGF_{165}$ wild type and all VEGF mutants in acute wound fluid, no degradation was detectable after 240 minutes.

Figure 2 shows the effect of chronic wound fluid on the stability of the VEGF proteins. Incubation of the VEGF wild type synthesized in COS-1 cells (Figure 2A, lane 1-4) for 240 minutes shows degradation of the growth factor with a fragment of about 38 kDa. This corresponds to the 110 dimer fragment of the less glycosylated VEGF variant.
In contrast to the wild type, the $VEGF_{165}$ mutants show a different degradation behaviour on incubation in chronic wound fluid. On the one hand, the degradation process observed in the mutations (Figure 2B, lanes 13-16 and Mut_{Ala} (lanes 5-8) is comparable to that of the wild-type. Fragments with a molecular weight of about 38 kDa are produced after only about 20 min.
On the other hand, analysis of the mutants Mut_{Pro} (lanes 9-12) and $Mut_{Lys\text{-}Pro}$ (Lanes 1-4, 17-20) shows a breakdown behaviour different from the wild type and the mutants Mut_{Ala} and Mut_{Gln}. Stable signal at 42 and 45 kDa is seen in the SDSPAGE up to 60 minutes after incubation. This indicates stabilization of the mutated proteins Mut_{Pro} and $Mut_{Lys\text{-}Pro}$ in the chronic wound fluid. This difference in the degradation behaviour of the mutants with neutral/nonpolar amino acid and those with proline suggests that further proteases, besides plasmin, are involved in the breakdown of VEGF in the chronic wound environment.

Degradation is observable with all mutated proteins 240 minutes after incubation in chronic wound fluid. In these cases there is not just formation of clearly defined breakdown fragments; on the contrary, a diffuse signal between 38 and 45 kDa appears after 240 min. This presumably involves proteolysis in the region of the first 20 amino acids (recognition site of the antibody), because the signal strength decreases markedly after 240 min.

In summary, the results indicate that the VEGF mutants with proline at position 111 are initially stabilized in chronic wound fluid but are degraded in the long term, Comparable results were observed in the would fluids from three different patients with chronic venous insufficiency. The experiments for the various wound fluids were repeated at least twice

(Figure 2B: patient X lanes 1-4; patient Y lanes 17-20). The resulting band pattern always remained the same moreover.

Figure 2C shows a densitometric evaluation of the breakdown of VEGF wild-type and $Mut_{Lys\text{-}Pro}$. The aim of the investigation was to quantify the stabilization of the VEGF mutant in the chronic wound fluid. For this purpose, the time-dependent change in the signal strength at the level of the initial signal (42-45 kDa region) compared with the signal at time zero was determined. The densitometric densities measured at the various times are depicted as percentage of the initial signal. It is clear in this densitometric investigation that at every measurement time the VEGF mutant shows a stronger signal by comparison with the VEGF wild type in the 42-45 kDa region, and thus intact $VEGF_{165}$ protein is present. This observation suggests that this mutation leads to an improved stability and bioactivity of the VEGF protein in the chronic wound environment. The difference between wild type and mutant is statistically significant only 20 minutes after the incubation. The measurements were carried out with identical wound fluid for three independent experiments.

II. Investigations of the biological activity of $VEGF_{165}$ wild-type and the mutated variants:

It was investigated whether the mutations have an effect on the biological activity of the VEGF molecule. The biological activity was assayed by means of a BrdU proliferation assay (Roche Diagnostics, Mannheim) on human umbilical vein endothelial cells (HUVE cells) in accordance with the manufacturer's information. This entailed the HUVE cells being cultivated with addition of various VEGF mutants, then incubated with BrdU solution for 6 hours and fixed, after which an ELISA was carried out using a BrdU-specific antibody.

VEGF concentrations between 1 ng/ml and 25 ng/ml were employed. Commercially available recombinant $VEGF_{165}$ protein (R&D Systems) and $VEGF_{165}$ wild-type synthesized in COS-1 cells showed a half-maximum stimulation of BrdU incorporation at about 3 ng/ml (Figure 3). The mutated VEGF proteins are characterized by a stimulation of endothelial cell proliferation which is comparable to the VEGF wild type synthesized in COS-1 cells. The maximum stimulation of all the proteins synthesized in COS-1 cells was less than that by commercially obtainable $VEGF_{165}$ wild-type. The reason for the difference between the two curved profiles may be the different expression systems and

purification methods for the proteins (Mohanraj *et al.* 1995). The biological activity of $VEGF_{165}$ is thus not significantly affected by the mutations carried out.

The question of the extent to which the biological activity of the $VEGF_{165}$ wild type and of the VEGF mutants is affected after plasmin incubation was subsequently examined. For this purpose, the VEGF proteins were incubated with plasmin, and then the biological activity was investigated by means of a BrdU proliferation assay on HUVE cells.

In the graphical representation (Figure 4), the BrdU incorporation is shown as percentage of the initial signal (time t = 0). Incubation of the VEGF wild-types (synthesized in COS-l cells and from R&D Systems) and of the VEGF mutants Mut_{Ala} and $Mut_{Lys\text{-}Pro}$ in plasmin buffer at 37°C shows no impairment of the biological activity of the proteins (Figure 4A-D). In contrast thereto, incubation of the $VEGF_{165}$ wild-types in plasmin leads to a marked reduction in the biological activity (Figure 4A, B). An activity loss of at least 20% is seen only 20 minutes after incubation, and then falls further to about 50% of the initial activity after 240 minutes. The Mut_{Ala} and $Mut_{Lys\text{-}Pro}$ mutants show no significant activity loss after incubation with plasmin (Figure 4C, D). These results underline the "plasmin resistance" of the mutants demonstrated in the Western blot (Figure 1) and show that the mutated proteins are stable even after incubation with plasmin.

The introduced mutations thus result in an inhibition of VEGF cleavage by plasmin. A stabilization of VEGF and thus an increased biological activity in the chronic wound environment can be brought about by the Ala_{111} to Pro_{111} mutation.

Figure 1: The $VEGF_{165}$ mutations are resistant to cleavage by plasmin. The figure shows incubation of $VEGF_{165}$ and the mutated proteins in a plasmin solution [0.01 U/ml] or buffer solution (B, lanes 18, 19) for the stated periods. Analysis of the degradation behaviour took place by Western blotting and immunodetection.

Figure 2: The Ala_{111} to Pro_{111} mutation increases the stability of VEGF in chronic wound fluid. A) $VEGF_{165}$ wild-type expressed in COS-l cells and B) the VEGF variants were incubated in chronic wound fluid for the stated periods, and the degradation behaviour was visualized by immunodetection. In this case, wound fluids from two different patients were investigated: patient X, lanes patient Y: lanes 17-20). C) Densitometric visualization

of the degradation of VEGF wild-type and in chronic wound fluid. The relative signal strength from three independently performed Western blot analyses (mean +/- SD) is shown.

Figure 3: The VEGF mutants are biologically active. $VEGF_{165}$ wild-type and VEGF mutants were each incubated in increasing concentrations with HUVE cells. The rate of incorporation of the base analogue into the DNA of the proliferating cells determined by BrdU ELISA is shown (mean +/- SD; n=3).

Figure 4: Plasmin does not alter the biological activity of the $VEGF_{165}$ mutants. A comparison is shown of the relative BrdU incorporation into HUVE cells through stimulation with $VEGF_{165}$ wild type (A, B), Mut_{Ala} (C) and $Mut_{Lys\text{-}Pro}$ (D) after incubation of the stated protein in buffer or plasmin (means +/-SD; n = 3).

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Scharffetter-Kochanek, Meewes C, Eming S, et al., Z. Hautkrankheiten 74:239-249, 1999.

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Sequence Protocol

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Val Asp Ile Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys
        35                  40                  45

Pro Ser Cys Val Pro Leu Met Arg Cys Gly Gly Cys Cys Asn Asp Glu
    50                  55                  60

Gly Leu Glu Cys Val Pro Thr Glu Glu Ser Asn Ile Thr Met Gln Ile
65                  70                  75                  80

Met Arg Ile Lys Pro His Gln Gly Gln His Ile Gly Glu Met Ser Phe
                85                  90                  95

Leu Gln His Asn Lys Cys Glu Cys Arg Pro Lys Lys Asp Arg Pro Arg
            100                 105                 110

Gln Glu Asn Pro Cys Gly Pro Cys Ser Glu Arg Arg Lys His Leu Phe
        115                 120                 125
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CLAIMS

1. A vascular endothelial growth factor (VEGF) variant, characterized in that the alanine at AA position 111 is replaced by proline.

2. The VEGF variant according to claim 1, characterized in that the arginine at AA position 110 is replaced by another amino acid.

3. The VEGF variant according to claim 1 and/or 2, wherein the VEGF variant is present in the form of one of the splice variants $VEGF_{121}$, $VEGF_{145}$,$VEGF_{165}$,$VEGF_{183}$,$VEGF_{189}$ or $VEGF_{206}$.

4. $VEGF_{165}$ variant according to one of the claims 1 to 3, characterized in that they comprise one of the amino acid sequences

Seq. No. 1:
Ala Pro Met Ala Glu Gly Gly Gly GIn Asn His His Glu Val Val Lys Phe Met Asp Val Tyr Gin Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lvs Pro Ser Cys Val Pro Leu Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys Glu Cys Arg Pro Lys Lys Asp Arg **Pro** Arg Gln Glu Asn Pro Cys Gly Pro Cys Ser Glu Arg Arg Lys His Leu Phe Val Gln Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser Arg Cys Lys Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr Cys Arg Cys Asp Lys Pro Arg Arg

or Seq. No. 2:
Ala Pro Met Ala Glu Gly Gly Gly Gln Asn His Glu Val Val Lys Phe Met Asp Val Tyr Gln Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His Gln Gly Gln

His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys Glu Cys Arg Pro Lys Lys Asp **Lys Pro** Arg Gln Glu Asn Pro Cys Gly Pro Cys Ser Glu Arg Lys His Leu Phe Val Gln Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser Arg Cys Lys Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr Cys Arg Cys Asp Lys Pro Arg Arg.

5. VEGF variant according to one of the claims 1 to 4, characterized in that the amino acid sequence is modified or derivatized and/or does comprise mutations, insertions and/or deletions, and/or does comprise a signal sequence.

6. VEGF-variant according to claim 5, characterized in that the signal sequence signal sequence is N-terminally connected to the amino acid chain of the VEGF variant and has the sequence

Met Asn Phe Leu Ser Trp Ser Val His Trp Ser Leu Ala Leu Leu Leu Tyr Leu His His Ala Lys Trp Ser Gln Ala.

7. Nucleic acids coding for VEGF variants according to one of the claims 1 to 6.

8. Vectors comprising nucleic acids according to claim 7 for the expression of VEGF variants according to one of the claims 1 to 6.

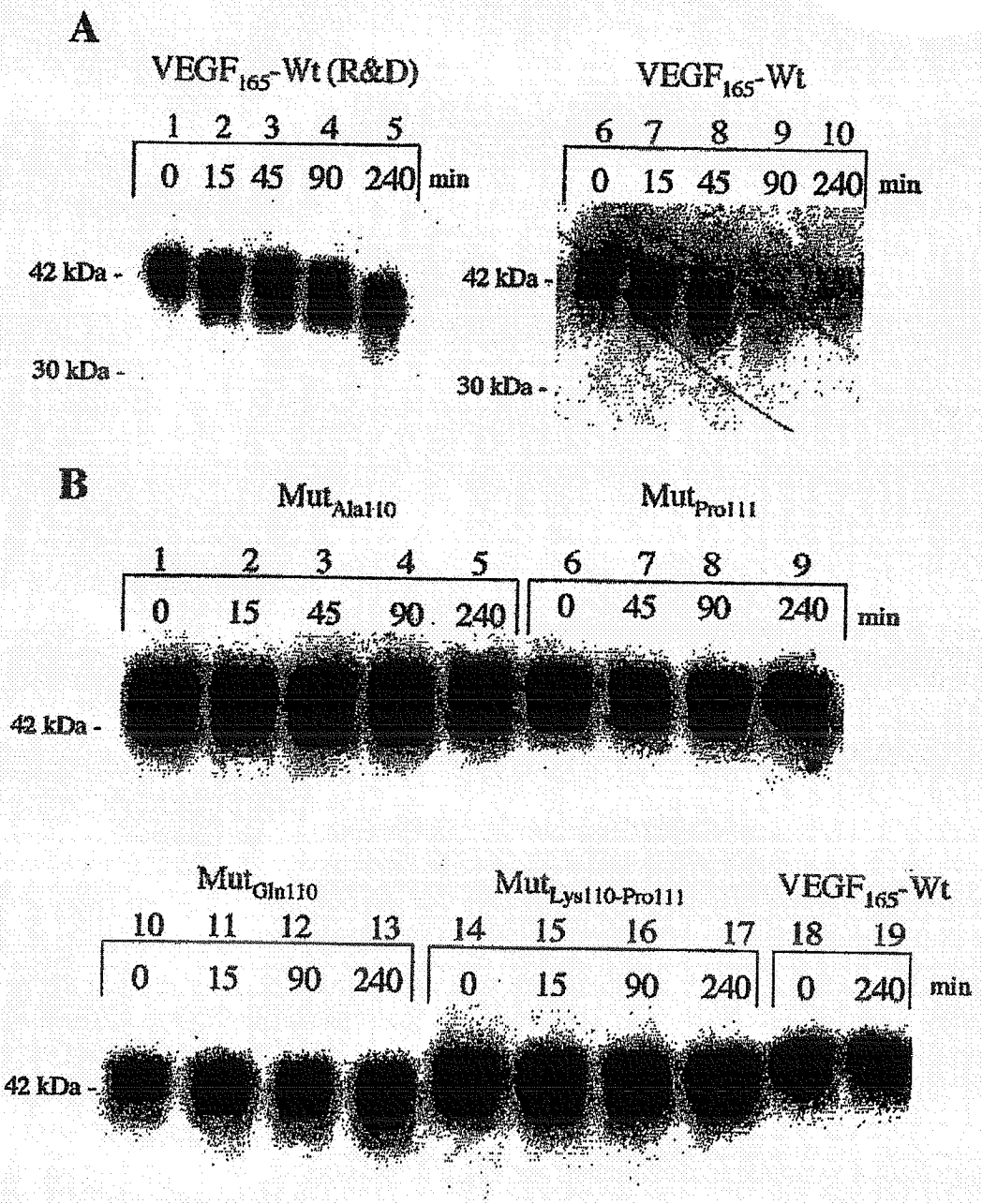
9. Medicament comprising VEGF variants according to one of the claims 1 to 6, nucleic acids according to claim 7 or vectors according to claim 8.

10. Use of VEGF variants according to one of the claims 1 to 6, of nucleic acids according to claim7 or vectors according to claim 8 for the production of a medicament for the treatment of chronic wounds, especially caused by vascular lesions, such as chronic venous insufficiency (CVI), primary/secondary lymphoedema, arterial occlusive disease, metabolic disorders, such as diabetes mellitus, gout or decubitus ulcer, chronic inflammatory disorders, such as pyoderma gangrenosum,

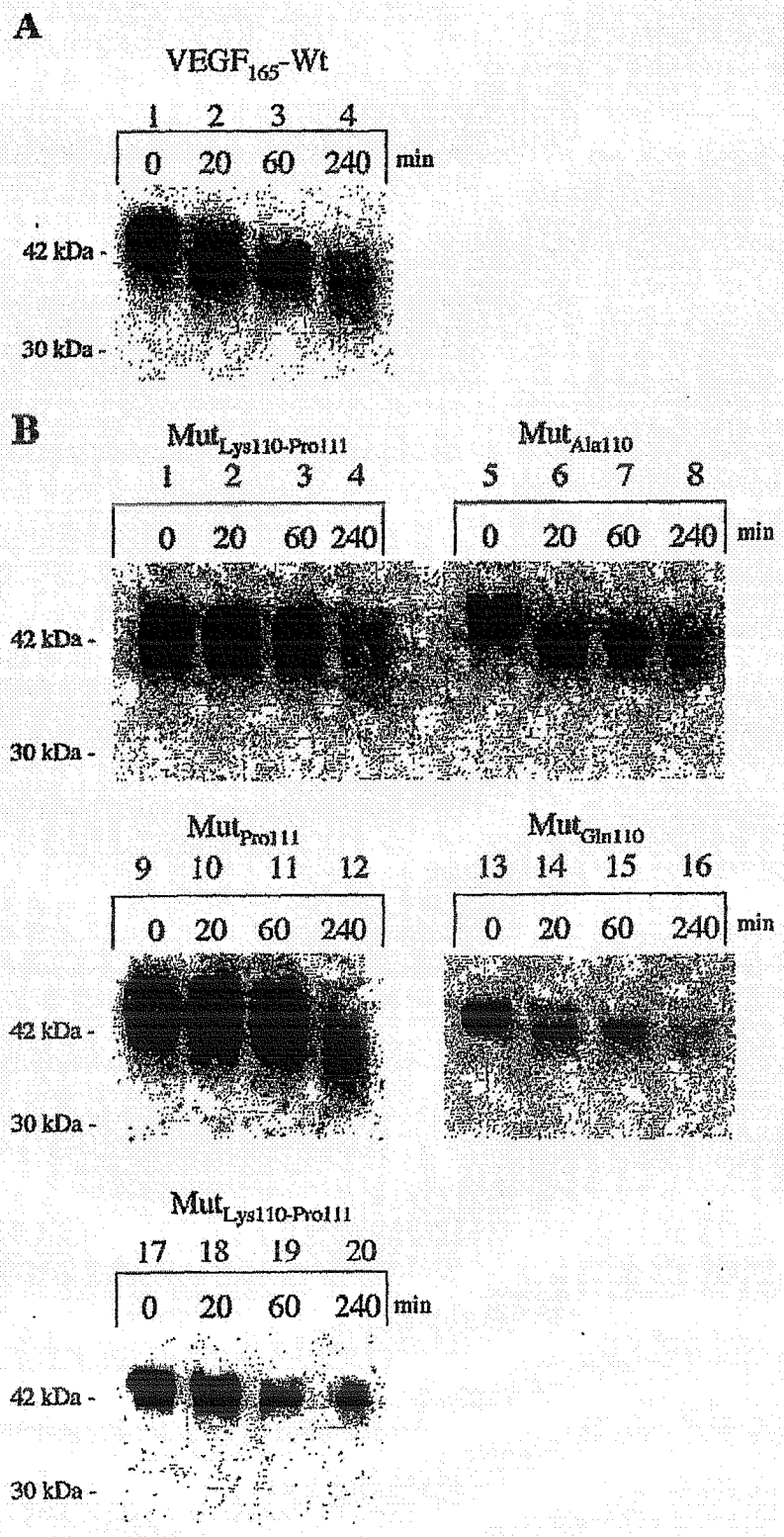
vasculitis, perforating dermatoses, such as diabetic necrobiosis lipoidica and granuloma annulare, haematological primary disorders such as coagulation defects, sickle-cell anaemia and polycythemia vera, tumours, such as primary cutaneous tumours and ulcerative metastases, for plasmin inhibition, for the induction of neoangiogenesis, and/or for the inhibition of matrix degradation.

Summary

The invention relates to an endothelial growth factor (VEGF) in which the alanine at amino acid (AA) position 111 is replaced by proline and the arginine at AA position 110 can be substituted by another amino acid. The invention also relates to derivatives of the inventive VEGF, nucleic acids, expression systems, medicaments, and the use of the VEGF mutants of the invention for treating chronic wounds.


A
VEGF165-Wt (R&D)
1 2 3 4 5
0 15 45 90 240 min
42 kDa -
30 kDa -
VEGF165-Wt
6 7 8 9 10
0 15 45 90 240 min
42 kDa -
30 kDa -
B
Mut Ala110
Mut Pro111
1 2 3 4 5 6 7 8 9
0 15 45 90 240 0 45 90 240 min
42 kDa -
Mut Gln110
Mut Lys110-Pro111
VEGF165-Wt
10 11 12 13 14 15 16 17 18 19
0 15 90 240 0 15 90 240 0 240 min
42 kDa -

Figur 1


A
VEGF165-Wt
1 2 3 4
0 20 60 240 min
42 kDa -
30 kDa -
B
MutLys110-Pro111
1 2 3 4
0 20 60 240
MutAla110
5 6 7 8
0 20 60 240 min
42 kDa -
30 kDa -
MutPro111
9 10 11 12
0 20 60 240
MutGln110
13 14 15 16
0 20 60 240 min
42 kDa -
30 kDa -
MutLys110-Pro111
17 18 19 20
0 20 60 240 min
42 kDa -
30 kDa -

Figur 2 A, B

C

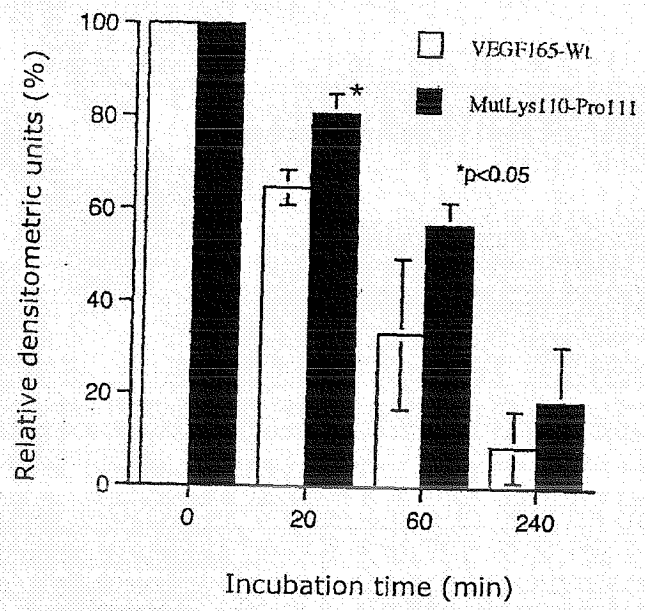

100
80
60
40
20
0
Relative densitometric units (%)
VEGF165-Wt
MutLys110-Pro111
*p<0.05
*
0
20
60
240
Incubation time (min)

Figure 2C

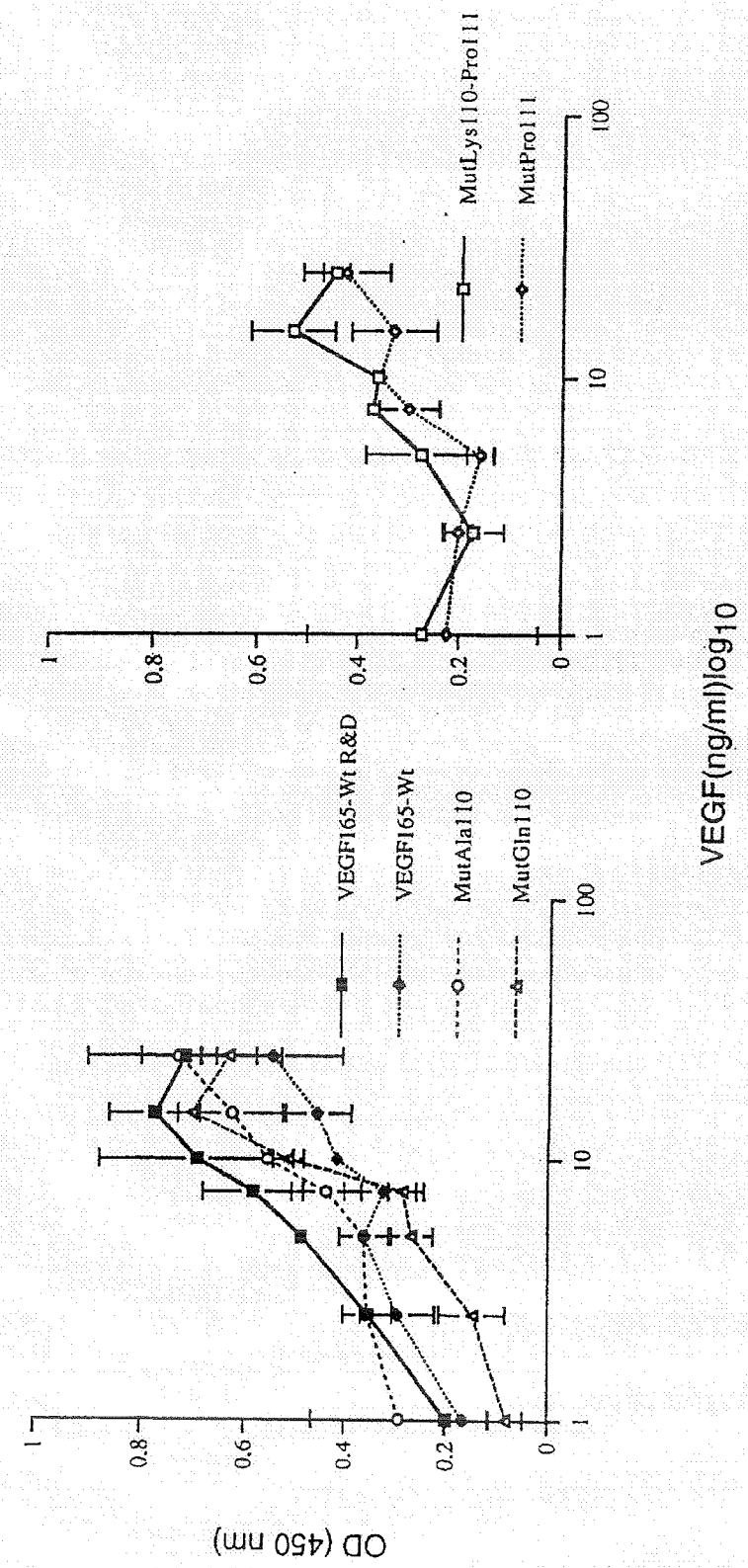

OD (450 nm)
VEGF(ng/ml)log10
VEGF165-Wt R&D
VEGF165-Wt
MutAla110
MutGln110
MutLys110-Pro111
MutPro111
0
0.2
0.4
0.6
0.8
1
10
100

Figure 3

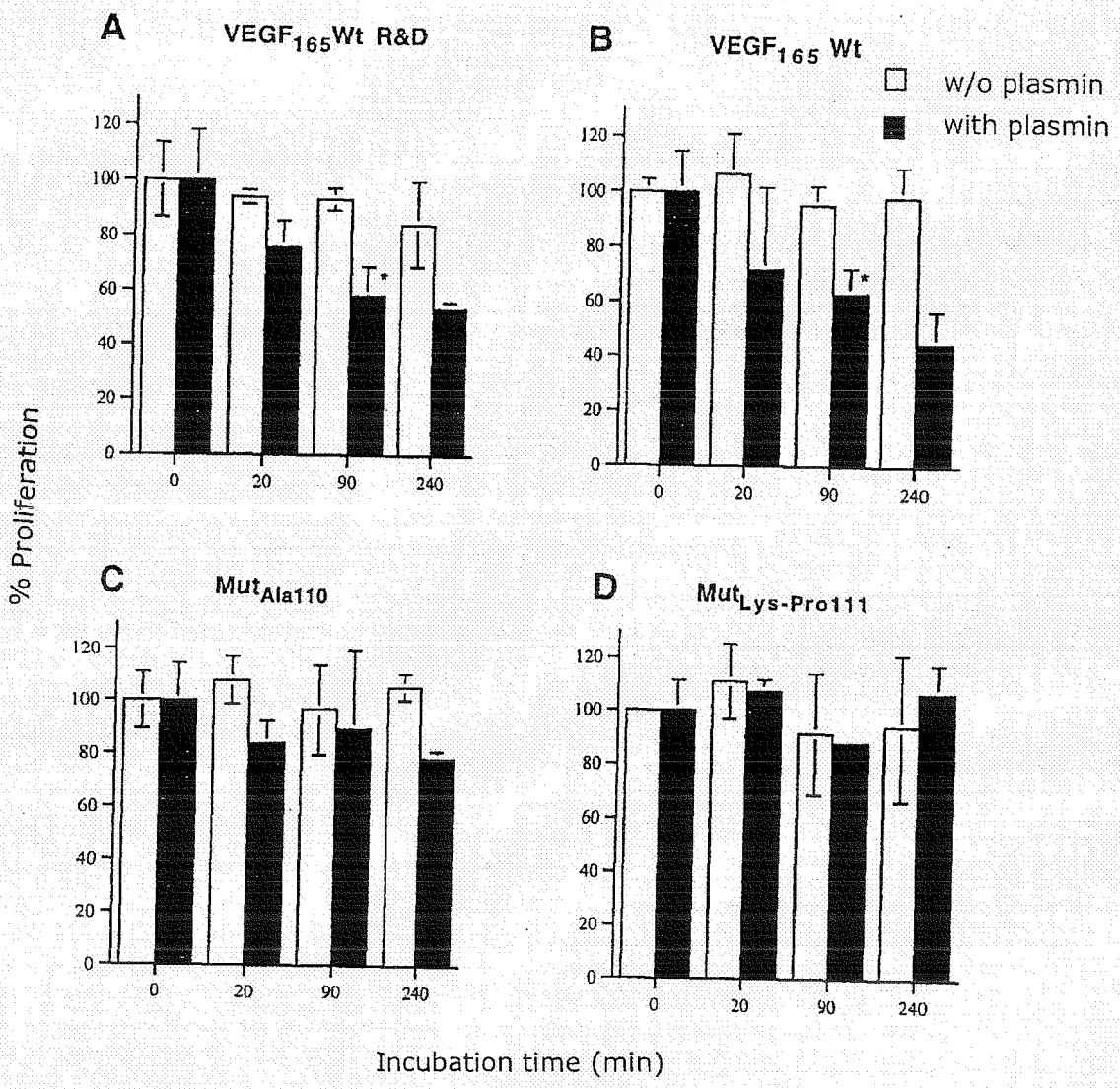

A
VEGF165Wt R&D
B
VEGF165 Wt
w/o plasmin
with plasmin
C
MutAla110
D
MutLys-Pro111
% Proliferation
Incubation time (min)
0
20
90
240
120
100
80
60
40
20
0
*

Figure 4